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Mutations in the *draT* and *draG* Genes of *Rhodospirillum rubrum* Result in Loss of Regulation of Nitrogenase by Reversible ADP-Ribosylation

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Reversible ADP-ribosylation of dinitrogenase reductase forms the basis of posttranslational regulation of nitrogenase activity in *Rhodospirillum rubrum*. This report describes the physiological effects of mutations in the genes encoding the enzymes that add and remove the ADP-ribosyl moiety. Mutants lacking a functional *draT* gene had no dinitrogenase reductase ADP-ribosyltransferase (DRAT, the *draT* gene product) activity in vitro and were incapable of modifying dinitrogenase reductase with ADP-ribose in vivo. Mutants lacking a functional *draG* gene had no dinitrogenase reductase-activating glycohydrolase (DRAG, the *draG* gene product) activity in vitro and were unable to remove ADP-ribose from the modified dinitrogenase reductase in vivo. Strains containing polar mutations in *draT* had no detectable DRAG activity in vitro, suggesting likely cotranscription of *draT* and *draG*. In strains containing *draT* and lacking a functional *draG*, dinitrogenase reductase accumulated in the active form under derepressing conditions but was rapidly ADP-ribosylated in response to conditions that cause inactivation. Detection of DRAT in these cells in vitro demonstrated that DRAT is itself subject to posttranslational regulation in vivo. Mutants affected in an open reading frame immediately downstream of *draTG* showed regulation of dinitrogenase reductase by ADP-ribosylation, although differences in the rates of ADP-ribosylation were apparent.

Rhodospirillum rubrum, a purple, nonsulfur, photosynthetic bacterium, has a well-characterized system of posttranslational regulation of its nitrogenase by ADP-ribosylation. The nitrogenase complex consists of two proteins: dinitrogenase, an $\alpha_2\beta_2$ tetramer of the *nifK* and *nifD* gene products, contains the active site of nitrogenase, and dinitrogenase reductase, an α_2 dimer of the *nifH* gene product, supplies the reducing power to dinitrogenase. Regulation of the complex is achieved by reversible mono-ADP-ribosylation of dinitrogenase reductase at Arg-101 (23) in response to light level and a fixed nitrogen concentration (12).

Two enzymes have been shown to perform this regulation in vitro. Dinitrogenase reductase ADP-ribosyltransferase (DRAT, the gene product of *draT*) transfers an ADP-ribose from NAD to one subunit of dinitrogenase reductase (18, 20), thereby inactivating nitrogenase by preventing electron flow within the nitrogenase complex (22). Dinitrogenase reductase-activating glycohydrolase (DRAG, the gene product of *draG*) removes the ADP-ribosyl group from dinitrogenase reductase, restoring activity to the nitrogenase complex (21, 28–30). The *draT* and *draG* genes have been isolated and sequenced (7) and are adjacent to, but transcribed in the opposite direction from, the *nifHDK* genes (15).

The first description of mono-ADP-ribosylation was that of eucaryotic elongation factor 2 by diphtheria toxin (11). Subsequently, a number of other bacterial toxins, including cholera toxin and pertussis toxin, have been shown to act by ADP-ribosylating proteins in eucaryotic cells (reviewed in references 8 and 33). This has led to the suggestion that endogenous regulatory systems also may operate by ADP-ribosylation of specific target proteins. ADP-ribosyltransferases capable of modifying proteins and ADP-ribosylglycohydrolases capable of cleaving ADP-ribose from model proteins have been isolated from eucaryotic cells (35). A number of endogenous, reversible ADP-ribosylation systems have also been reported in procaryotes (19 and references therein).

Construction of mutations in the *draT* and *draG* genes and biochemical characterization of the resulting mutants are described in this report. The phenotypes of these mutants were used to test the current model for regulation of nitrogenase by DRAT and DRAG (23) and provide insight into DRAT and DRAG regulation.

MATERIALS AND METHODS

Growth of bacteria. The *R. rubrum* strains used in this study are listed in Table 1. They were grown at 30°C on malate-ammonium medium (MN) or supplemented (yeast extract) malate-ammonium medium (SMN) and derepressed for nitrogenase on malate-glutamate medium (MG), a modification of Ormerod's medium (3, 26), as described elsewhere (7, 12). A spontaneous streptomycin-resistant mutant (strain UR2) (7) was used for all conjugation experiments and was used as the wild-type control for tests in vivo. For experiments using kanamycin-resistant (Kan^r) strains, 10 μg of kanamycin per ml was added to the medium. Kanamycin always was used as the sulfate salt. For studies using protein synthesis inhibitors, 15 μg of chloramphenicol per ml was added to the medium. *Escherichia coli* strains were grown at 37°C on LB medium (24).

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TABLE 1. Strains and plasmids used in this study[a]

Allele	Strain	Plasmid used for construction	Relevant characteristic	DRAG activity[b]	DRAT activity[c]
dra$^+$	UR2		Wild type	60	0.004
draT2	UR212	pJHL217	*draT* polar	<2	<0.0004
draT3	UR213	pJHL220	*draT* nonpolar	38	<0.0004
draG4	UR214	pJHL201	*draG* polar	<2	0.002
draG5	UR215	pJHL214	*draG* nonpolar	NT[d]	NT
draG6	UR220	pJHL302	*draG* polar	<2	0.003
ORF	UR216	pJHL222	ORF polar	14[e]	0.005

[a] All mutant alleles are from this work.
[b] Nanomoles of ethylene produced per milligram of protein per minute in a fixed-time nitrogenase assay.
[c] Picomoles of ADP-ribose incorporated per milligram of protein per minute. The assay is precise to ±10%.
[d] NT, not tested.
[e] The total DRAG activity in strain UR216 was approximately 35% of that of the wild type. The value presented here is the activity of membrane-associated DRAG; DRAG activity was apparently distributed anomalously between the soluble and pellet fractions in the extract of the ORF mutant.

Construction of mutations in cloned regions. Plasmid DNA was isolated by using the alkaline lysis method (1). Specific DNA fragments generated by restriction endonuclease digestion were isolated by using a Geneclean kit (BIO101, LaJolla, Calif.) after separation of the fragments by agarose gel electrophoresis. Other DNA manipulations were performed by standard methods (24).

Plasmid pWPF102 (7) contains the genes *draT* and *draG*, an adjacent open reading frame (ORF), and 0.47 kb of the downstream region on a 2.7-kb *Sma*I fragment (Fig. 1). Plasmid pJHL200 was constructed by replacing the *Eco*RV-*Pvu*II fragment containing most of the tetracycline resistance element of pSUP202 (31), which does not replicate in *R. rubrum*, with the 2.7-kb *Sma*I fragment of pWPF102. This construction lacks the *dra* promoter, as the site of *Sma*I cleavage is only 14 bp upstream of the translation start codon (TTG) of *draT* (7). A 1-kb *Acc*I-*Sac*II fragment containing the *dra* promoter and part of *draT* (from pWPF102) was subcloned into pJHL200 by replacing the *Cla*I-*Sac*II fragment, creating pJHL210. In addition, plasmid pJHL300 was constructed by replacing the *Eco*RV-*Pvu*II fragment of pSUP202 with the 7.8-kb *Bam*HI fragment containing the *dra* promoter, the *dra* genes, and all of the downstream region from pWPF102.

The Kan^r cassette, which contains a transcriptional terminator and translational stop codons at each end, was cut out of pHP45ΩKm (6) with *Eco*RI. After the ends were filled with T4 DNA polymerase, the cassette was inserted into both pJHL200 and pJHL210 at the *Eco*RV site in *draG*, creating pJHL201 and pJHL211, respectively. The same cassette fragment was inserted into pJHL210 at the *Sac*II site in *draT* (after blunt ends were formed at this site with the Klenow fragment of DNA polymerase) and at the *Hin*cII site in the ORF to create pJHL217 and pJHL222, respectively. The Kan^r cassette from pUC4K was inserted into a Δ*Sal*I site in *draG* on pJHL300 to create pJHL302.

Nonpolar insertion mutations were constructed as follows. The Kan^r cassette was removed from pJHL211 by *Bam*HI digestion and religation, creating pJHL214. Removal of the cassette left a 24-bp fragment (which encodes an amino acid sequence of Asn-Ser-Arg-Gly-Ser-Pro-Gly-Ile) of the original cassette sequence between Asp-82 and Ile-83 of the DRAG sequence. The cassette in pJHL217 was removed by *Sma*I digestion and religation, creating pJHL220. This removal resulted in replacement of Arg-191 of the DRAT sequence with the amino acid sequence Glu-Phe-Pro-Gly-Ile. For cloning convenience, a selectable Kan^r marker from pUC4K (34) was inserted at the *Pst*I site in both pJHL214 and pJHL220.

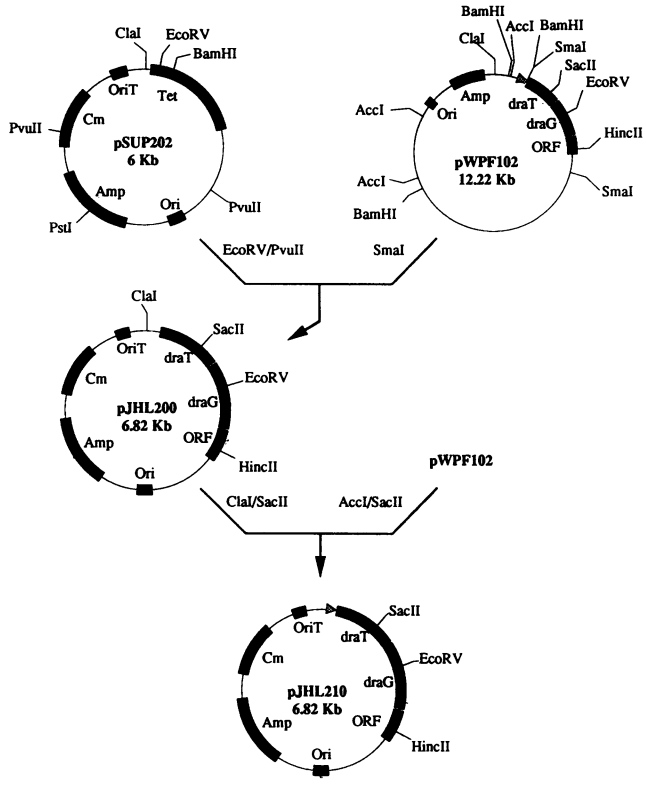

FIG. 1. Generation of plasmids used in this study. Only restriction sites used in the constructions are indicated. The constructions are described in Materials and Methods. The hatched arrow represents the *dra* promoter.

Reintroduction and resolution of the *dra* mutations. The constructed plasmids were mobilized from *E. coli* into *R. rubrum* by conjugation as follows (17). Equal volumes (0.1 ml) of a mid-log-phase culture of *E. coli* S17-1 containing the constructed plasmid and a fresh overnight culture of *R. rubrum* were mixed gently in a microcentrifuge tube. The cells were centrifuged, the supernatant was decanted, and the pellet was resuspended in about 10 μl of the medium remaining in the tube. The cells were spread on a sterile membrane filter (25 mm, 0.45-μm pore size) on an SMN plate and incubated at 30°C for 24 h. The cells were then resuspended by vortexing in 1 ml of MN medium, diluted in MN medium, and plated onto MN plates containing 50 μg of kanamycin per ml.

In experiments in which polar insertions in the *dra* region were to be selected, the Kan^r colonies were replica plated onto MN plates containing 50 μg of kanamycin per ml and MN plates containing 50 μg of kanamycin per ml plus 10 μg of chloramphenicol per ml. To obtain pure *R. rubrum* cultures, the Kan^r, chloramphenicol-sensitive colonies were picked and streaked onto SMN plates containing 50 μg of kanamycin per ml. For isolation of strains containing nonpolar insertions, the Kan^r colonies, resulting from integration of the plasmid, were picked and reisolated from SMN plates containing 50 μg of kanamycin per ml. The isolates

were then grown in liquid SMN medium at 30°C for 72 h to allow resolution of the merodiploid. The liquid culture was diluted and plated on SMN plates and then replica plated on SMN plates and SMN plates plus 50 μg of kanamycin per ml. Kan^s colonies resulted from recombination between the mutant and wild-type *dra* regions, causing the vector and one *dra* allele to be recombined out of the chromosome and lost.

Verification of the mutations. The derived *dra* mutants were identified by their acquisition of restriction sites in the introduced DNA. Total chromosomal DNA was prepared from *R. rubrum* strains as previously described (7). After being digested with restriction enzymes overnight and separated by electrophoresis on a 0.6% agarose gel, the DNA was transferred to a nylon membrane and then hybridized to oligolabeled [^{32}P]DNA probes as described previously (10, 24).

Growth of *dra* mutants and treatment with NH_4Cl and darkness. Growth of the cells for in vivo experiments paralleled the method of Kanemoto and Ludden (12). Cells of mutants and wild-type *R. rubrum* were grown in 5 ml of SMN medium (supplemented with 10 μg of kanamycin per ml for Kan^r mutants) in aerobic, screw-top test tubes. After an initial period of cell growth, 15 ml of MN medium was added and the test tubes were sealed. Illuminated, water-jacketed 500-ml vessels, continuously flushed with helium and containing MG medium, were inoculated with the 20-ml starter cultures. Samples were taken periodically to monitor the growth and nitrogenase activity of the cells. When the cells reached an A_{600} of approximately 1.0, either the light source was turned off or an O_2-free solution of NH_4Cl (1 mM final concentration) was added. Cultures used for ^{32}P studies were grown in the presence of 1 mCi of $H^{32}PO_4^{2-}$ per culture.

Nitrogenase assay in vivo. Nitrogenase activity was measured by the acetylene reduction method (5). Two milliliters of cells was withdrawn anaerobically from the culture vessel and injected into a 21-ml stoppered serum vial containing a gas phase of 10% acetylene and 90% N_2. The vial was placed in an illuminated, shaking water bath at 30°C for 2 min, after which time 0.5 ml of 25% trichloroacetic acid was added. A sample of the gas in the vial was analyzed for ethylene by flame ionization after gas chromatography on a Porapak N column. Nitrogenase activity is reported as nanomoles of ethylene produced per milliliter of cells per hour.

Immunoprecipitation and visualization of radiolabelled dinitrogenase reductase. Approximately 10 ml of a 1.0-A_{600} cell culture was collected by filtration and frozen rapidly in liquid nitrogen to minimize changes in the ADP-ribosylation state of dinitrogenase reductase (12). The frozen samples were homogenized to lyse the bacteria, and dinitrogenase reductase was immunoprecipitated with specific antibodies. The ADP-ribosylated subunit of the reductase was then separated from the unmodified subunit by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10% [wt/vol] acrylamide total; 0.13% [wt/wt] bisacrylamide cross-linker) by the method of Laemmli (14) with modifications (12). Autoradiography showed the presence or absence of radiolabelled dinitrogenase reductase.

Partial purification of DRAG. Approximately 5 g of cell paste from cultures grown on MN medium was lysed by osmotic shock (21). The chromatophore membranes were separated by centrifugation at 250,000 × *g* and then washed with buffer containing 0.5 M NaCl. Under these conditions, DRAG is released from the membranes.

DRAG activity assays. Extracts containing DRAG were incubated with reductant, dinitrogenase, and excess inactive dinitrogenase reductase in a nitrogenase assay mixture for 15 min. Activation of dinitrogenase reductase was then monitored by coupling to the acetylene reduction activity of nitrogenase (30). DRAG activity is reported as nanomoles of ethylene produced per milligram of protein per minute.

DRAT activity assays. Approximately 1 g of cell paste from cultures grown on either MN or MG medium was disrupted by sonication. Ten microliters of the crude extract was incubated with active dinitrogenase reductase and [α-^{32}P] NAD for 20 min, after which time the mixture was precipitated, collected onto filters, and counted in a liquid scintillation counter (18). DRAT activity is reported as picomoles of ADP-ribose incorporated per milligram of protein per minute.

Immunoblotting. Protein samples of DRAG were electrophoresed on SDS-polyacrylamide gels containing 11% (wt/vol) acrylamide (total; 5% [wt/wt] bisacrylamide cross-linker) (14, 18) and then electroblotted to a nitrocellulose membrane. Binding of the antibodies and visualization with alkaline phosphatase were done by the method of Blake et al. (2) with modifications (4).

RESULTS

Construction and physiological characteristics of mutants. A series of mutants containing either polar or nonpolar insertions in the *dra* region and the downstream ORF were generated. Proper chromosomal incorporation of both polar and nonpolar insertions was confirmed by DNA hybridization experiments and, in the strains with nonpolar mutations, by the appearance of additional restriction enzyme sites. No obvious differences between the mutants and wild-type *R. rubrum* were noted as regards their growth on various media or the appearance of the cultures, in contrast to previous attempts to introduce *dra* mutations into *R. rubrum* (7).

Nitrogenase activity in the mutants. The level of nitrogenase activity of mutants derepressed on MG medium was comparable to that of the wild type (Fig. 2, 3, and 4, time 0′). It was surprising that the *draG* mutants had nearly normal levels of nitrogenase activity, since these mutants have a functional *draT* gene. This result is addressed in the Discussion.

Phenotype of mutants in response to darkness and ammonium. When treated with a light-dark-light cycle, wild-type *R. rubrum* (12) and strain UR2 showed rapid loss of nitrogenase activity in the dark and then reappearance of that activity in the light (Fig. 2A). UR213, the strain with the nonpolar *draT3* mutation, showed no loss of whole-cell nitrogenase activity when assayed after a dark treatment. Repeated cycles of darkness and light also showed no change in nitrogenase activity (Fig. 2B). Strain UR212, containing the polar *draT2* mutation, behaved similarly (data not shown). For strain UR2, addition of NH_4Cl caused loss of activity, while NH_4Cl addition had no effect upon UR213 (Fig. 2) or UR212 (data not shown).

The modification status of dinitrogenase reductase was determined at various time points by immunoprecipitating samples of a culture grown on $H^{32}PO_4^{2-}$ as described previously (12). Samples from a culture of UR2 showed the appearance and removal of radiolabel from dinitrogenase reductase, coincidental with the loss and gain of nitrogenase activity measured in vivo (Fig. 5A). Samples from UR212 and UR213 showed no incorporation of [^{32}P]ADP-ribose into dinitrogenase reductase under any of the conditions tested (data not shown), verifying that dinitrogenase reductase was

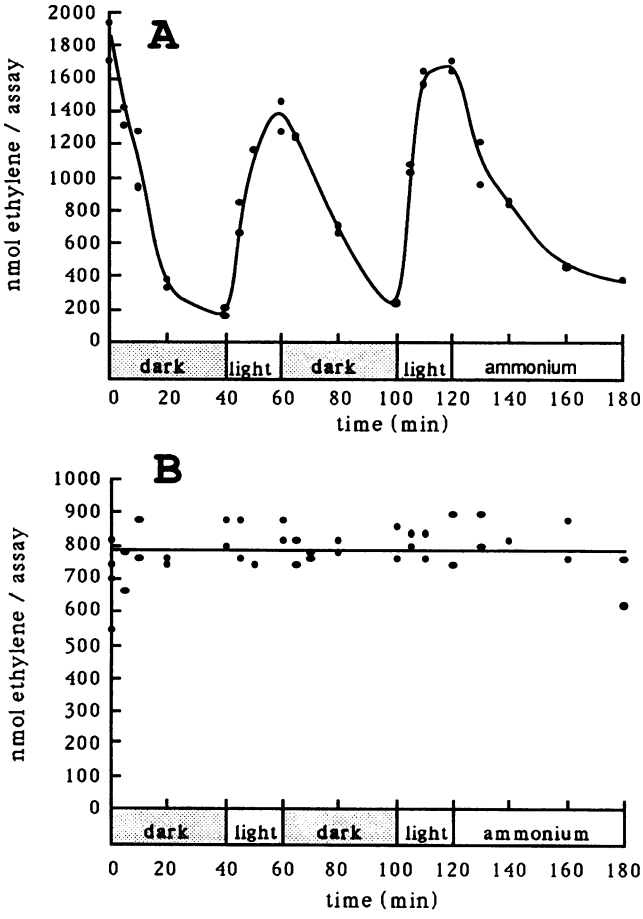

FIG. 2. Response of the wild-type and a *draT* mutant to inactivation conditions. *R. rubrum* UR2 (wild type) (A) and UR213 (*draT*) (B) were derepressed for nitrogenase, and at the time points indicated, duplicate 2-ml samples were withdrawn and immediately assayed for nitrogenase activity (ethylene produced) for 2 min in the light. As shown at the bottom of each panel, the cultures were exposed to two cycles of 40 min of darkness and 20 min of illumination, followed by addition of NH_4Cl to a final concentration of 1 mM.

not ADP-ribosylated in response to darkness or NH_4Cl in these mutants.

As noted above, *draG* mutants UR214 and UR220 produced active nitrogenase in vivo during normal derepression even though DRAT should be present in these cells. The following experiments demonstrate that DRAT activity is detectable in vivo when cells are shifted to inactivating conditions, thus providing evidence for posttranslational regulation of DRAT. Figures 3 and 5B show that dinitrogenase reductase in these mutants became ADP-ribosylated upon removal of the culture from light or addition of NH_4Cl to the culture. Samples from cultures grown in the presence of $H^{32}PO_4^{2-}$ showed inactivation of dinitrogenase reductase by [^{32}P]ADP-ribosylation upon addition of NH_4Cl or removal from the light source (Fig. 5B). As the loss of nitrogenase activity appears faster in this mutant, the radiolabel associated with dinitrogenase reductase before the start of the experiment (Fig. 5B, lane 1) may represent partial inactivation due to darkness during sampling. Reillumination of the culture did not result in rapid return of nitrogenase activity or removal of radiolabel from dinitrogenase reductase, although activity did begin to return after a lag of several hours. The return of nitrogenase activity in these mutants apparently was due to new synthesis of dinitrogenase reductase, as cultures exposed to chloramphenicol 30 min before the cells were placed in darkness did modify dinitrogenase reductase but did not exhibit this increase in nitrogenase activity upon reillumination.

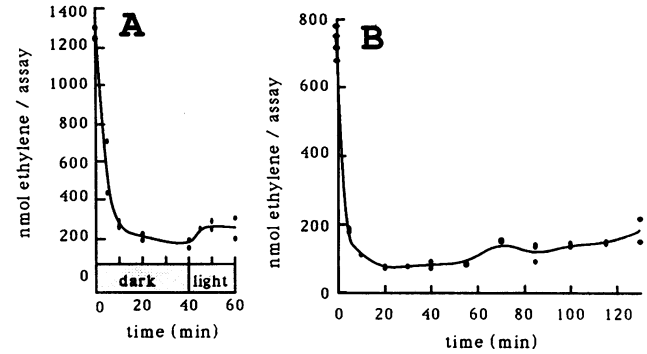

FIG. 3. Response of a *draG* mutant to inactivation conditions. *R. rubrum* UR214 (*draG*) was derepressed for nitrogenase, and acetylene reduction activity was assayed as described in the legend to Fig. 2. As shown at the bottom of panel A, the culture was exposed to a cycle of 40 min of darkness and 20 min of illumination. A second culture (B) was treated with NH_4Cl at a final concentration of 1 mM.

UR216 contains a mutation in the ORF downstream of *draG* and exhibits a pattern of regulation similar to that of the wild type during cycles of illumination and darkness and after NH_4Cl addition (Fig. 4 and 5C). The small differences

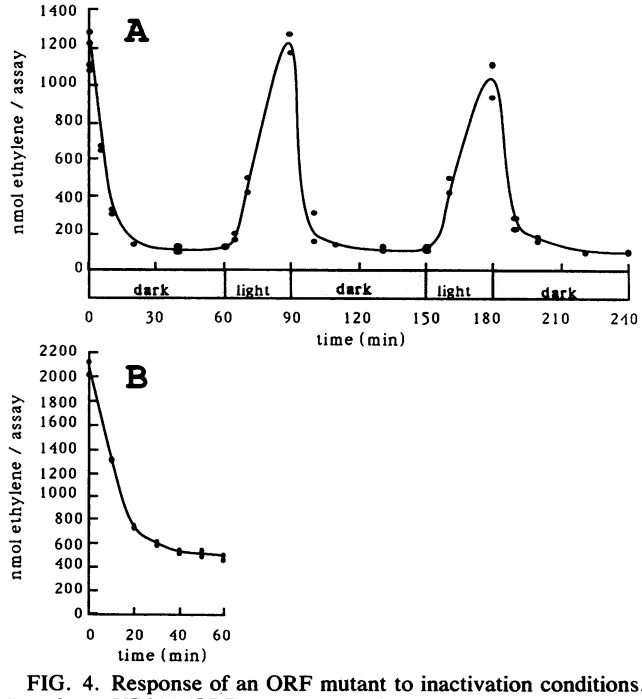

FIG. 4. Response of an ORF mutant to inactivation conditions. *R. rubrum* UR216 (ORF mutant) was derepressed for nitrogenase, and acetylene reduction activity was assayed as described in the legend to Fig. 2. As shown at the bottom of panel A, the culture was exposed to two cycles of 60 min of darkness and 30 min of illumination, followed by 60 min of darkness again. A second culture (B) was treated with NH_4Cl at a final concentration of 1 mM.

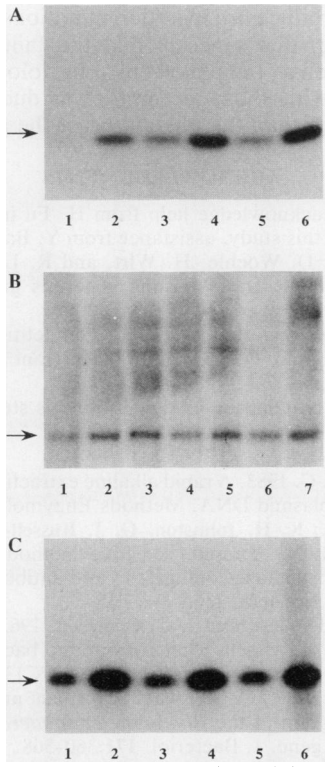

FIG. 5. Radioactivity incorporated into dinitrogenase reductase after growth of various strains of *R. rubrum* on $H^{32}PO_4^{2-}$. (A) Strain UR2 (wild type), treated as described in the legend to Fig. 2A (darkness from 0 to 40 min, light from 40 to 60 min, darkness from 60 to 100 min, light from 100 to 120 min, and NH_4Cl from 120 to 180 min). Lanes: 1, at 0 min; 2, at 40 min; 3, at 60 min; 4, at 100 min; 5, at 120 min; 6, at 180 min. (B) Strain UR214 (*draG*), similar to Fig. 3A but treated for longer times (darkness from 0 to 60 min, light from 60 to 120 min, and darkness again from 120 to 180 min). Lanes: 1, at 0 min; 2, at 30 min; 3, at 60 min; 4, at 90 min; 5, at 120 min; 6, at 150 min; 7, at 180 min. (C) Strain UR216, treated as described in the legend to Fig. 4A (darkness from 0 to 60 min, light from 60 to 90 min, darkness from 90 to 150 min, light from 150 to 180 min, and darkness from 180 to 240 min). Lanes: 1, at 0 min; 2, at 60 min; 3, at 90 min; 4, at 150 min; 5, at 180 min; 6, at 240 min. The arrows indicate the position of ADP-ribosylated dinitrogenase reductase.

in the rate of inactivation of nitrogenase in response to light-dark cycles, compared with the wild-type rate, are repeatable and may represent an alteration of control of DRAT and DRAG. Darkness appears to inactivate dinitrogenase reductase more rapidly in this mutant and may account for the radiolabel observed under conditions that should not cause modification (Fig. 5C, lanes 1, 3, and 5). In all other respects, the control of dinitrogenase reductase by ADP-ribosylation appears normal in strain UR216.

Evidence for the presence or absence of DRAT and DRAG in mutant strains. DRAG activity can be measured only in vivo in strains possessing DRAT, since ADP-ribosylated dinitrogenase reductase is the substrate of DRAG. To determine the presence of DRAG in strains lacking DRAT, partially purified samples of protein from mutant cultures, grown in either MN or MG medium, were analyzed by immunoblotting SDS-polyacrylamide gel electrophoresis samples with antibodies specific for DRAG (Fig. 6). DRAG protein was absent in strains with either a polar mutation in *draT* (UR212) or polar mutations in *draG* (UR214 and UR220). DRAG was present in the strain with a nonpolar

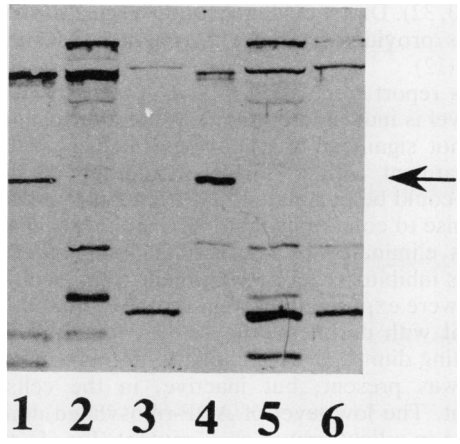

FIG. 6. Immunoblots using polyclonal antibody prepared against *R. rubrum* DRAG protein in the wild type and various mutants of *R. rubrum*. Lanes: 1, extract of MG-grown wild-type cells; 2, extract of MN-grown UR212 (*draT* polar) cells; 3, extract of MN-grown UR213 (*draT* nonpolar) cells; 4, DRAG standard; 5, extract of MG-grown UR214 (*draG* polar) cells; 6, extract of MN-grown UR216 (ORF polar) cells. The arrow indicates the position of DRAG protein.

mutation in *draT* (UR213) and in the strain with the ORF insertion (UR216), although the amount of DRAG protein was reduced in these strains relative to that of wild type. The absence of DRAG in the polar *draT* mutant is therefore suggestive of transcriptional polarity of *draT* on *draG*. In vitro activity assays for DRAG confirmed the absence of DRAG activity in UR212, UR214, and UR220 and the presence of activity in UR213 and UR216 (Table 1). Similarly, activity assays for DRAT confirmed the absence of DRAT activity in UR212 and UR213 and the presence of DRAT activity in all of the other strains (Table 1).

DISCUSSION

The in vivo roles of DRAT and DRAG as the agents of regulation of nitrogenase by reversible ADP-ribosylation in *R. rubrum* have been confirmed. Previous studies have shown that expression in *Klebsiella pneumoniae* of the *draTG* genes from *R. rubrum* was sufficient to allow ADP-ribosylation of nitrogenase in that organism, which is not known to possess this form of regulation (9). In *R. rubrum* mutants lacking DRAT, dinitrogenase reductase is not modified after cells are removed from the light or upon addition of NH_4Cl to the medium; thus, inactivation of nitrogenase is mediated in vivo exclusively by DRAT. Similarly, in mutants lacking DRAG, ADP-ribosylated dinitrogenase reductase cannot be activated; thus, activation of nitrogenase is mediated solely by DRAG in vivo.

An additional layer in the complex regulation of nitrogenase is also reported here. The possibility of posttranslational regulation of DRAT and DRAG activities, rather than transcriptional or translational control, was suggested by the observation that active DRAT and DRAG enzymes were found in extracts from a variety of cell types, including aerobically grown cells, *nif*-repressed anaerobically grown cells, and *nif*-derepressed cells that were competent for ADP-ribosylation. Further evidence for posttranslational regulation was the finding of these active enzymes in extracts from ammonium-starved, *nif*-derepressed cells that synthesize but do not regulate nitrogenase by ADP-ribosy-

lation (20, 32). Direct evidence for the regulation of DRAG in vivo was provided by the experiments of Kanemoto and Ludden (12).

In this report, regulation of DRAT activity at the enzymatic level is indicated by the fact that dinitrogenase reductase is not significantly ADP-ribosylated in *draG* mutants until treatment with darkness or addition of ammonium. While it could be postulated that DRAT is synthesized only in response to conditions that inactivate nitrogenase, such a model is eliminated by the results obtained with protein synthesis inhibitors. As noted, when strains with mutations in *draG* were exposed to protein synthesis inhibitors prior to treatment with darkness, they still were capable of ADP-ribosylating dinitrogenase reductase in vivo, indicating that DRAT was present, but inactive, in the cells prior to treatment. The low level of ADP-ribosylated dinitrogenase reductase in a light-grown *draG* mutant (Fig. 5) reflects the fact that the small amount of modification in these mutants that does occur is not removable because of the absence of DRAG activity. In addition, the ability of wild-type *R. rubrum* to perform several cycles of reversible ADP-ribosylation in the presence of protein synthesis inhibitors in vivo provides further evidence that *R. rubrum* does not control ADP-ribosylation of dinitrogenase reductase exclusively by regulation of DRAT and DRAG synthesis (data not shown).

This intracellular, posttranslational regulation of both DRAT and DRAG is lost in cell extracts. While both enzymes have specific requirements for activity in vitro, including nucleotide and metal ion levels, no correlation between these assay conditions and cellular levels of these components that explains the regulation of the DRAT-DRAG system has been demonstrated (13, 16, 25, 27).

Considerable (10 to 15%) residual nitrogenase activity always is observed in vivo under conditions in which nitrogenase should be inactivated in wild-type *R. rubrum*. This is also observed in strains UR214 and UR220, which demonstrates that the residual activity is not due to reactivation of the enzyme by DRAG and indicates that a portion of dinitrogenase reductase is not modifiable by DRAT, since inactive dinitrogenase reductase cannot support nitrogenase activity (22). We do not understand the nature of this resistance to modification.

The ability of DRAT to modify dinitrogenase reductase in vivo is more rapid in both the *draG* and downstream ORF mutants than in wild-type *R. rubrum*. This rapid inactivation of nitrogenase by DRAT, as well as the low level of ADP-ribosylation observed under conditions that should not cause modification of dinitrogenase reductase, may be caused by a decrease in DRAG activity in vivo. Both DRAG protein and DRAG activity are absent in the *draG* mutants, and their levels in the downstream-ORF mutant are lower than in the wild type. The reason for this decrease in DRAG levels in the ORF mutant is unknown, but the decrease may represent a change in protein stability or synthesis of the *draT* and *draG* gene products, and it also may represent the loss of signal transduction to, or a change in the control of, DRAT and DRAG at the enzymatic level.

While this report describes the successful construction of *dra* mutants in *R. rubrum*, our previous attempts to introduce *dra* insertion mutations into the chromosome were unsuccessful, leading to the speculation that such mutations were deleterious under the conditions used (7). Those previous attempts used an introduction system based on the incompatibility of replication-proficient IncP plasmids, which subsequently was shown to work poorly in this organism (17). Because the growth conditions used during all attempts to introduce *dra* insertion mutations into *R. rubrum* were the same, this suggests that the failure of previous attempts, as well as the pleiotropic physiological alterations observed following those attempts, was due to the method employed rather than the phenotype of the mutants.

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